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RESEARCH ARTICLE

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A comparison study on the cow and mare milk-clotting activity of *Withania coagu-*

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ABSTRACT

The limitations of rennin application in cheese crafting usually urge the discovery of novel proteases. The *Withania coagulans* fruits are well-known for their caseinolytic activity in cheese production. The study aims to evaluate some of the factors affecting the milk-clotting activity (MCA) of *W. coagulans* fruit enzymatic extract in even and odd-toed hoofed-mammals milk. The extracts were prepared by distilled water and normal saline and their protein content were evaluated. The time necessary for the appearance of discernible and discrete particles in the mare and cow milk by the two concentrations of saline and hydro *W. coagulans* extracts (SE and HE, respectively) was assessed at 35 and 40°C while exposing different levels of calcium chloride. The interaction of these factors on MCA was evaluated using mixed-design ANOVA. Three significant interaction patterns considering the maximum number of factors were revealed ($p < 0.05$). The higher extract concentration and incubation temperature (40°C) was always effective in producing the utmost MCA in these interactions. The SE was faster than HE in milk clot formation. The cow milk was a more suitable substrate than mare milk for the enzyme activity.

Keywords

Withania coagulans, milk-clotting activity, cow milk, mare milk

Abbreviations

MCA: Milk-clotting activity
E1: 25% concentration of the enzymatic extract
E2: 100% concentration of the enzymatic extract
HE: Hydro extract of *Withania coagulans*
SE: Saline extract of *Withania coagulans*

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Introduction

Cheese production is one of the most important methods for preserving the main ingredients of milk [1]. The enzymatic gelatinization is a crucial stage in cheese manufacturing [2]. Mostly, specific proteases gelatinize and form the cheese clots by protein coagulation and the release of whey. Currently, animal, herbal, fungal, and microbial proteases are applied along with the recombinant enzymes in cheese manufacturing [3].

The most commonly known cheese rennet originates from an animal source and is being extracted from suckling calf abomasum. Despite the benefits of this kind of rennet and its growing demand, its production has decreased drastically due to the less young calves slaughtering, rising meat prices, the emergence of dangerous diseases such as mad cow disease and the increase of vegetarianism [4, 5].

Microbial rennets reduce cheese yield and produce bitter peptides due to the high and non-specific proteolytic activity [6]. Genetically engineered cheese rennets also have limitations for consumers, which are prohibited in Germany, the Netherlands, and France [7].

Considering the limitation of renin production and its rising prices, plant proteases can be a good alternative to renin. Traditionally, plant rennets have been used in cheese production for long times [7]. Recently, studies postulate that plant proteases can be an affordable substitute for other rennets. Despite extensive researches on the possibility of plant rennet application in the cheese industry, some results indicate that these rennets are sometimes unsuitable for cheese making due to high proteolytic activity and a bitter taste of the final product. Furthermore, non-specific casein hydrolysis and the reduction of curd strength in comparison with calf chymosin is another negative prospect of plant rennets [8, 9].

One of the plant proteases is originated from *W. coagulans*, and it seems that it does not have the undesirable properties of plant rennets [10, 11]. The zymographic analysis of this enzyme has shown that it is an aspartic protease with a molecular weight of about 35 Kilo-dalton [12]. *W. coagulans*, a plant belonging to the Solanaceae family, grows wild all over Pakistan as well as southwest of India and Afghanistan. In Iran, the distribution of this plant is limited to Sistan and Baluchestan Province [13, 14].

Unlike ruminants' milk, mare milk does not usually produce a firm clot after the addition of common rennets [15]. Therefore, introducing an enzyme with a proper mare milk clotting activity is important in the cheese industry. Various factors affect the rennet coagulation properties. Most of the coagulants act at 35-40°C to form semi-hard to hard cheese [16].

In addition to the type and concentration of a milk-clotting enzyme, calcium chloride ($CaCl_2$) has been shown to enhance the formation of casein clots [17, 18]. Elevation of Ca^{2+} and calcium phosphate colloids along with the hydrogen ion activity are the main reasons for this enhancement [19].

The object of this study was to evaluate the role of type (saline and hydro) and concentrations of *W. coagulans* enzymatic extracts in commencing the clot particles formation in even and odd-toed ungulates' milk (cow and mare) at different incubation temperatures and $CaCl_2$ levels.

Results

The total extracted protein from *W. coagulans* in saline was higher than the distilled water. The protein contents of the HE and SE assessed via UV spectrophotometer absorbance were 78.62% and 57.63%, respectively.

The total protein and casein content of the mare and cow milk were evaluated by a formol titration method and a Kjeldahl-linked assay (Figure 1). Holstein cow milk had an average total protein of 3.6%. In this study, the total protein of Holstein cow milk was 1.9% higher than Arabian mare milk. Furthermore, the total casein of cow milk was 2.9% which results in a 1.5% higher casein content than the mare milk.

In this study, the simple effects of 5 factors, i.e.: milk type (cow vs mare), the level of $CaCl_2$ in milk (0.5, 10, and 200mM), the type (HE vs SE) and the concentration (E1 and E2) of *W. coagulans* extract and the reaction temperature (35 and 40°C) were investigated on the start of milk clot formation (Figure 2). Considering the maximum number of factors, three

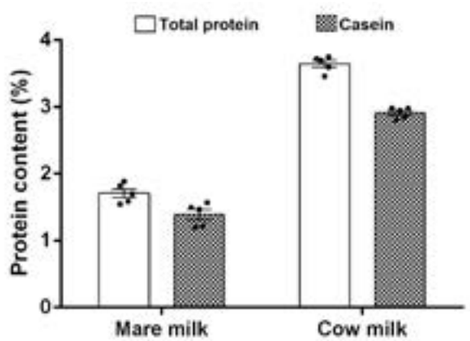

Figure 1.
The total and casein proteins concentration of the cow and mare milk (mean ± SEM; n = 5).

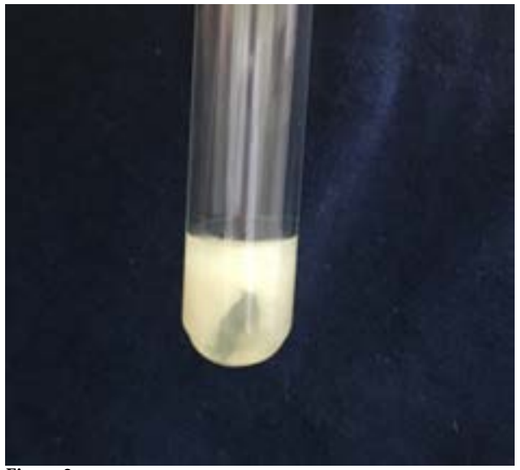

Figure 2.
The milk clot results from the *W. coagulans* extract.

significant interaction patterns (each containing four factors at the same time) were identified which affect the MCA.

In the first pattern, the four factors which comprise milk type, $CaCl_2$ level, *W. coagulans* extract concentration, and reaction temperature showed interaction with each other (p = 0.00025). Hence, the MCA was affected by the different levels of these four factors. The highest MCA level was 1824 units, which was observed due to the effect of 100% concentration of the extract on cow milk in the presence of 200 mM $CaCl_2$ at 40°C. The statistical differences of the interaction between the levels of these four factors are shown in Figure 3.

The second interacting pattern is between the four factors: milk type, reaction temperature, type and concentration of *W. coagulans* extract (p = 0.016). In this interaction, the highest MCA level (1324 units) comes from the effect of SE with 100% concentration on the cow milk at 40°C. The statistical differences between the levels of these four factors are shown in Figure 4.

Finally, the four factors of reaction temperature, $CaCl_2$ level, and type and concentration of *W. coagulans* extract demonstrated the third interaction pattern (p = 0.045). In this interaction pattern, the highest MCA level was 1310 units and was observed due to the effect of 100% of SE in the presence of 200 mM $CaCl_2$ at 40°C. The statistical differences between the levels of these four factors are shown in Figure 5.

Discussion

In the simultaneous assessment of all the three interaction patterns, it is found that the factors of temperature and the extract concentration play more important roles because these factors are being effective in all three patterns. Despite the effect of factors such as extract type, $CaCl_2$ level and milk type on MCA in some patterns, there is always an interaction pattern without the significant effect of these factors on MCA. For example, the extract type in the first interaction pattern, the level of $CaCl_2$ in the second interaction pattern and the milk type in the third interaction pattern (shown in the results) were not significant.

In this study, elevating the temperature from 35°C to 40°C increased the MCA. This increase in the activity may be due to the enzyme or substrate molecules. As the temperature rises, the rate of enzymatic reaction can also be increased due to the elevated kinetic energy of the substrate molecules. The number of substrate molecules with a kinetic energy of more than the energy barrier for product formation increases at higher temperatures. High substrate kinetic energy also increases their motion, and eventually the collision frequency of the molecules [20]. Beigomi and colleagues also showed that the activity of *W. coagulans* enzyme increases up to the temperature of 70°C [21]. Each protein has its own temperature susceptibility. The higher MCA of the enzyme at 40°C may also pertain to its steady 3D structure at this temperature. Generally, enzymes lose their 3D structure at high temperatures which results in their inactivity. For example, the optimal temperature for the activity of *Aspergillus niger* protease on shrimp peptone is 50°C [22].

Typically, MCA is dependent on the concentration of available enzymes [23]. The enzyme concentration can increase the amount of Kappa-casein proteolysis [24]. In this study, the higher extract (enzyme) concentration in cow milk always lowered the coagulation time and increased MCA (Figures 3; 4-B). The high concentration of *W. coagulans* extract increased MCA in mare milk at 40°C (Figure 3). Accordingly, the interaction of extract concentration and the reaction temperature sometimes plays an important role in MCA. Several models have been proposed for milk coagulation time versus enzyme concentration. In the first model, clotting time is inversely proportional to enzyme concentration [25], in the second model, the inverse clotting time is proportional with the concentration of enzyme [26], and in the third model, milk-clotting time is proportional to the inversed second root of enzyme concentration [27]. The higher concentration of *W. coagulans* extract reduces the time of skim milk clotting similar to other milk-clotting enzymes, including animal rennets (chymosin and pepsin), fungal rennet (*Mucor miehei* and *Mucor pusillus*) and the plant enzymes such as *Cyanara*

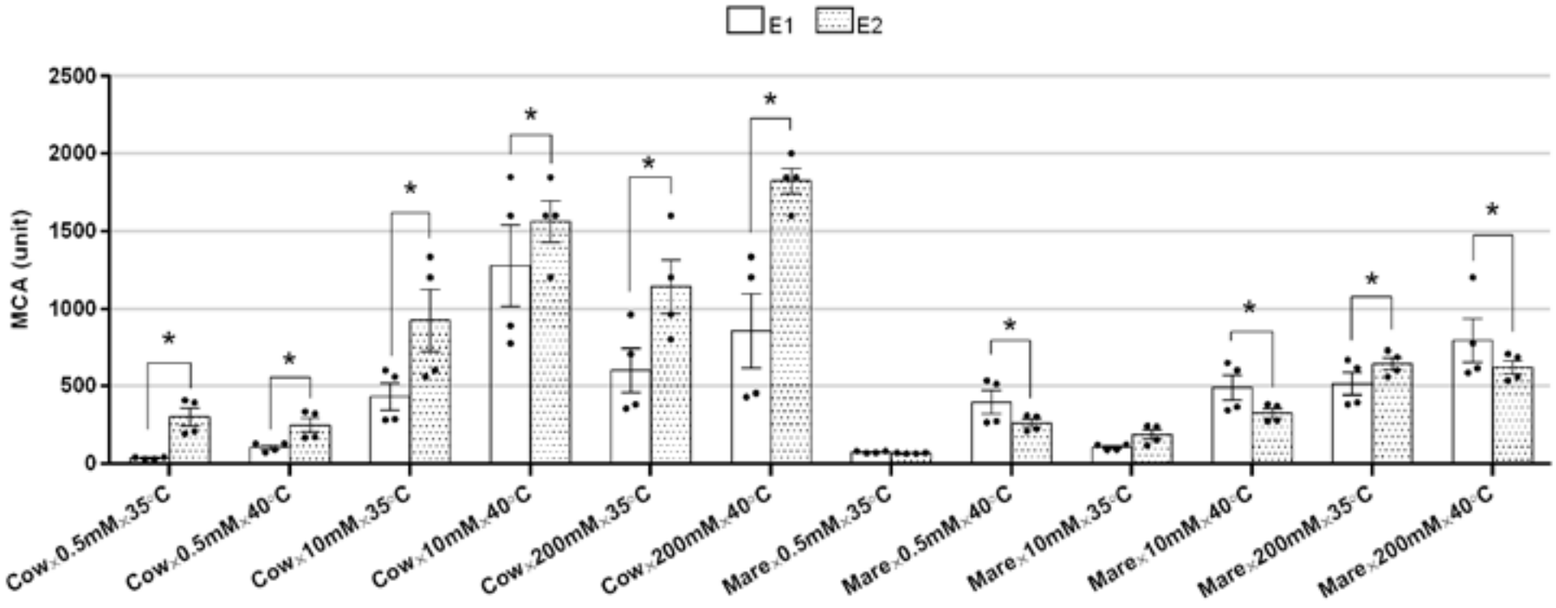

Figure 3
The MCA [mean±SEM; n=4] changes ($p < 0.05$) due to the concentrations of *W. coagulans* extracts (E1 and E2) in the presence of three $CaCl_2$ levels (0.5, 10, and 200 mM) in the mare and cow milk at 35 and 40°C.

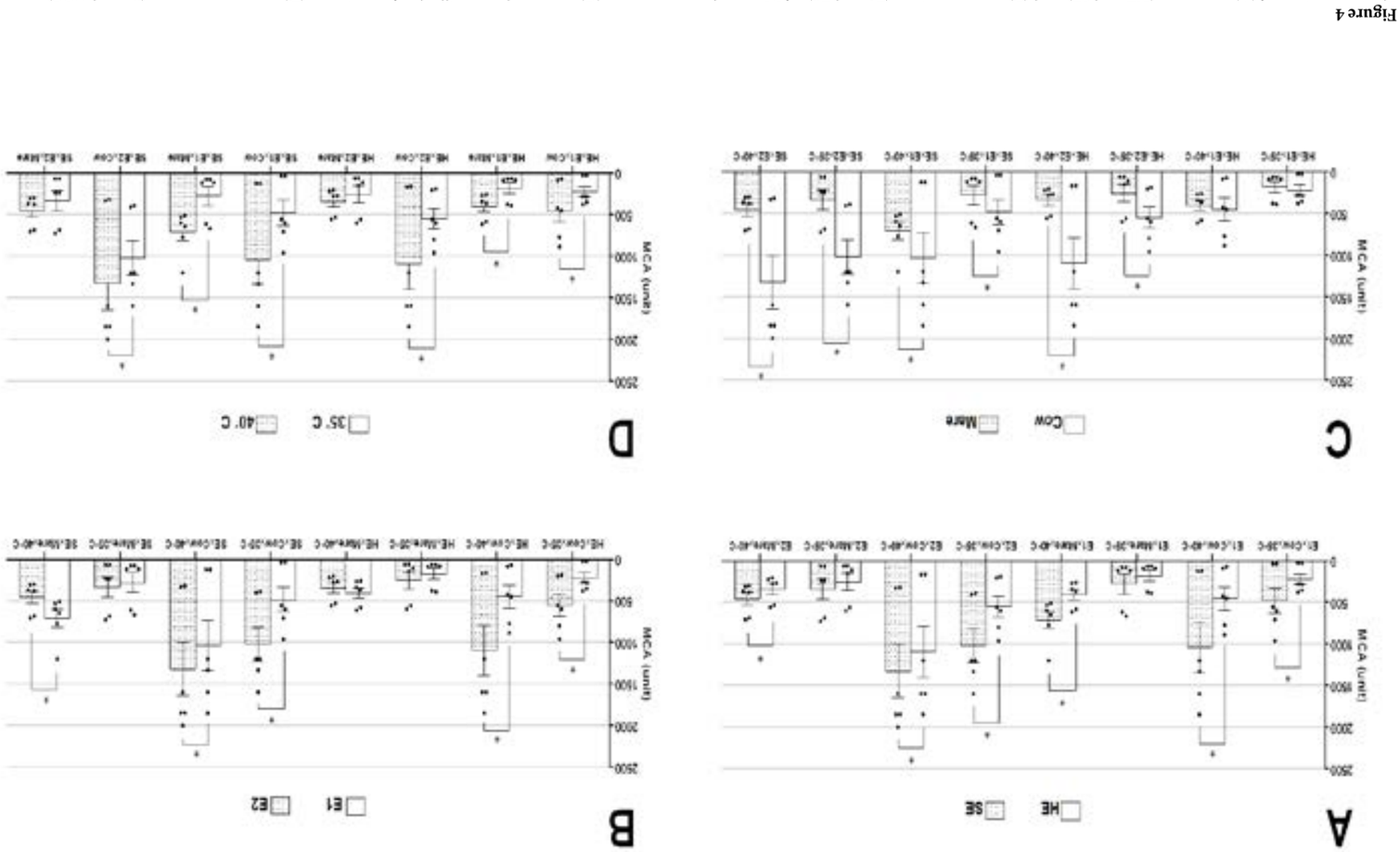

Figure 4
The interactions of (A) two types (HE and SE) and (B) concentrations (E1 and E2) of *W. coagulans* extracts on (C) mare and cow milk clot formation at (D) two temperatures (35 and 40°C) [mean±SEM; n=6] ($p < 0.05$).

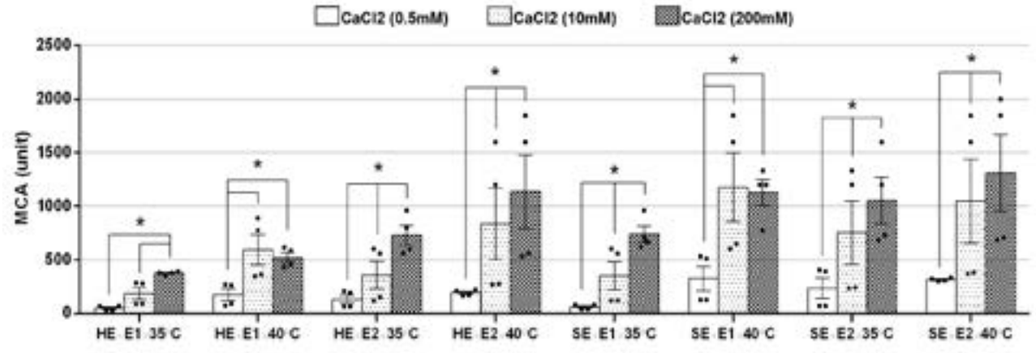

Figure 5.
The effect of three $CaCl_2$ levels (0.5, 10, and 200 mM) on the MCA [mean ± SEM; n=4] results of two types (HE and SE) and concentrations (E1 and E2) of *W. coagulans* extracts at 35 and 40°C ($p < 0.05$).

scolymus L. [23, 28, 29].

The SE always showed a higher clotting activity in the cow milk than the HE. While this extract in the mare milk exhibited more clotting activity than the HE only at 40°C (Figure 4-A). Some studies indicate that the protein's solubility increases at low salt levels [30]. For example, the solubility of the lysozyme protein increases in the physiological level of NaCl [31]. Chaotropic ions are known as 'salting-in' responsible ions due to their ability in increasing the solubility of proteins [32]. Divalent ions are more capable of protein salting-out than the monovalent ions (NaCl-forming ions) [33]. As shown, the higher protein content of SE than the HE may indicate a better enzyme extraction by normal saline. Traditionally and in some studies, saline is used to extract the enzyme of this plant for cheese production [34-36].

The milk type is effective on MCA in the first and second interaction patterns. Contrary to these two patterns, milk type does not play a role in the third interaction pattern (Figure 5). On many occasions, e.g.: SE and the high concentration of HE, the MCA in cow milk was higher than the mare milk (Figure 4). However, there was not any significant difference between the mare and cow milk clot formation by the 25% concentration of HE (Figure 4-C). The *W. coagulans* enzyme shows caseinolytic activity [12]. The amount of cow milk casein is higher than mare milk. The total mare milk proteins contain 40-60% casein. This proportion does not change extensively during the lactation period. Other dairy species such as cattle have higher casein levels in their milk [37]. The ratio of kappa-casein levels in mare milk is lower than ruminants [38]. Robitaille et al. showed that increasing the kappa-casein to total milk casein ratio accelerates cheese clot formation and enhances the clot consistency [39]. Furthermore, the temperature sensitivity of mare milk is lower than cow milk [40]. So that the cow milk at a higher temperature is more prone to clotting by the enzyme. Notably, in the evaluation of MCA in this study, milk type on some occasions (i.e.: the third interaction pattern) does not show any interaction with the temperature.

In this study, mostly the MCA enhanced as the level of $CaCl_2$ increased (Figure 5). $CaCl_2$ induces casein clot formation in milk through various mechanisms. It reduces the time for curd formation by changing the hydration forces between casein micelles and water [41]. Furthermore, increased H^+ and Ca^{2+} levels and their association with casein phosphate and carboxylic groups are needed to initiate clot formation [24]. The addition of $CaCl_2$ supplements reduces the time of clot formation by calf rennet, rennet-pepsin, *Mucor miehei* rennet and the *Bacillus sphaericus* clotting enzyme [42, 43]. Addition of 20–200 mM $CaCl_2$ in milk causes a 70°C heat-induced coagulation [44]. Here, there was no significant difference between the presence of 10 and 200 mM of $CaCl_2$ in MCA with a low concentration of the extracts at 40°C, whilst in other situations, the higher $CaCl_2$ level increased MCA (Figure 5). High levels of $CaCl_2$ might also increase casein clots production due to decreased sample pH [16]. Here, mostly the MCA was higher with 10 mM $CaCl_2$ rather than 0.5 mM $CaCl_2$. Adding 10mM $CaCl_2$ into milk enhances curd firmness about 81%. This is especially important in preventing weak casein clots [17].

In summary, it has been shown that saline (0.85%) extract of *W. coagulans* fruit has a higher protein content and higher MCA than the HE. The evaluations revealed that raising the temperature from 35°C to 40°C and increasing the extract concentration significantly elevates MCA. These factors have interactions

with other factors such as the type of milk, the type of extract and the $CaCl_2$ level on affecting the MCA level. The *W. coagulans* extract is likely to have more clotting activity on cow milk than mare milk due to its higher casein content and the casein's structure. Although the fruit extract is capable of cow milk clotting, the parameters such as the cheese yield and cheese sensory properties should be evaluated after using SE on different ruminants' milk at 40°C through cheese crafting studies.

Material and methods

Plant and milk preparation

The fruits of *W. coagulans* were prepared from Sistan and Baluchestan Province, Iran. The fruits were kept in a dark zip-sealed plastic bag in a cool place until the usage. Fresh cow milk was obtained from the Holstein breed and the fresh mare milk was from the Arab horse during the warm season. The two types of milk were pasteurized at 73°C for 15 seconds and cooled to 4°C. The milk was used in the assay within 2 days after pasteurization.

Preparation of W. coagulans extracts

The skin and pulp of *W. coagulans* fruits were carefully separated from the seeds. These compartments were mixed and powdered by mortar and pestle. The HE and SE of *W. coagulans* were extracted by distilled water and normal saline (0.85%). The fruit powder and the solvent (1:6) were mixed thoroughly in a shaking incubator (JSR, JSSI-100C compact shaking incubator) at 4°C for 24h. Homogenates were filtered (Whatman No. 1) and the filtrates were assayed immediately as the crude extracts [34].

Protein assessment of the W. coagulans extracts

The protein concentration of the extracts was evaluated by UV spectrophotometer absorbance [45]. Various dilutions of the HE and SE were prepared and their absorbance was measured at 260 and 280 nm spectrophotometrically (Shimatzo, Japan, UV-1201). The protein concentration of the extracts was calculated by Equation (1) as follows:

Equation (1): Protein concentration (mg/mL^{-1}) = (Abs.280 × 1.55) – (Abs.260 × 0.76)

Total protein and casein contents of cow and mare milk

Cow and mare milk total protein was evaluated via a titration method proposed by Pyne [46]. To assess the casein content of milk, 50 mL of warm water (40°C) and 0.5 mL of acetic acid solution (10%) were added chronologically to 5 g of milk. After 10 minutes, the milk blend was mixed with 0.5 mL of sodium acetate (1N) and allowed to cool at room temperature. The mixture was filtered through filter paper (Whatman No. 1) while its beaker was washed three times with distilled water. The filter paper containing casein precipitates was digested in a Kjeldahl digestion flask and its total nitrogen was further assessed via the Kjeldahl method. Casein content of the samples was calculated using 6.38 as the protein factor [47].

Preparation of cow and mare skim milk powder

The fresh milk was centrifuged at 4°C (5000 rpm / 15 min.) in falcon tubes (Universal 320R centrifuge, Hettich, Tuttlingen, Germany) and further defatted manually. This procedure was repeated twice and the skim milk was allowed to shadow-dry on a plate for 24h. The dried skim milk was powdered by mortar and pestle and kept at 4°C until usage within 7 days.

Evaluation of the extracts' MCA

The milk clot formation of HE and SE extrcats in cow and mare were measured at 35 and 40°C. The Arima et al, 1970 and Beigomi et al, 2014 methods were used to measure the MCA of the extracts with some modifications [21, 48]. Three different levels (0.5, 10 and 200 mM) of $CaCl_2$ (Merck, 64271) were mixed thoroughly with 10% reconstituted skim milk powders. Afterward, the reconstituted skim milk containing $CaCl_2$ was warmed to 35 or 40°C for 5 min in waterbath (Memmert WNB-29, Germany). Two concentrations (E1 and E2) of the crude HE and SE were prepared using their own solvents. The extract and the warmed reconstituted skim milk (1:10) were mixed gently. The time of the first visible milk clots formation was recorded for each experiment. Each experiment was performed at least in triplicates. The milk without the extract was considered as the negative control while distilled water and saline were also considered as the solvent control in which produced no clot at the aforementioned temperatures. The MCA of the extracts was calculated using the following formula (Equation 2):

Equation (2): MCA (units) = 2400/T × S/E

Where "T" is the time of clot formation (seconds), "S" represents substrate volume (mL) and "E" stands for the *Withania* enzymatic extract volume (mL).

Statistical analysis

After performing descriptive statistics, the interaction of different factors (milk types, extract types, extract concentrations, $CaCl_2$ levels, and incubation temperatures) on the MCA was evaluated using a mixed-design ANOVA with a significant level of 0.05. The Syntax results are presented with Bonferroni correction.

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Author Contributions

H.E. conceived and designed the experiments. H.E., F.H. and S.M. performed the experiments. H.E. and F.H. analyzed the data and wrote the manuscript.

Conflict of Interest

The authors declare that they have no conflict of interest.

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RESEARCH ARTICLE

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